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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>A61K 31/74, 35/14, 38/00, 38/16</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/17785</b> <b>(43) International Publication Date:</b> 15 April 1999 (15.04.99)
<b>(21) International Application Number:</b> PCT/US98/16659 <b>(22) International Filing Date:</b> 10 August 1998 (10.08.98) <b>(30) Priority Data:</b> 60/061,341 8 October 1997 (08.10.97) US <b>(71) Applicant (for all designated States except US):</b> THERAGEM, INC. [US/US]; 48-129 Bi-State Plaza, Old Tappan, NJ 07675 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> HOFFMAN, Brian, F. [US/US]; 109 Columbia Heights, Brooklyn, NY 11201 (US). BINAH, Ofer [IL/IL]; Nofit 148, 36001 Kiryat Tivan (IL). <b>(74) Agents:</b> ZITRON, Anne, E. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022-7513 (US).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> REPTILIAN-DERIVED PEPTIDES FOR THE TREATMENT OF MICROBIAL INFECTION  <b>(57) Abstract</b>  The present invention provides compositions useful as antimicrobial agents which include reptilian hemoglobin, the $\alpha$ and $\beta$ chains of hemoglobin free of heme, fragments of said proteins or polypeptide fragments thereof and combinations thereof. The compositions exert antimicrobial activity against both bacteria and fungi that is comparable to known antimicrobial peptides from human neutrophils, cathepsin G and azurocidin. Sensitive organisms include Gram-negative bacteria such as <i>Escherichia coli</i> and <i>Pseudomonas aeruginosa</i> , and the fungus <i>Candida albicans</i> . Methods for preparing the compositions also are provided.		

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## REPTILIAN-DERIVED PEPTIDES FOR THE TREATMENT OF MICROBIAL INFECTION

The present application claims the benefit of U.S. Provisional Application Ser.  
10 No. 60/061,341 filed on October 8, 1997, the entire disclosure of which is incorporated  
herein by reference.

### FIELD OF THE INVENTION

The present invention relates to a method for treating microbial infections of  
15 mammals, including humans and other primates; a method for killing bacteria and fungi;  
and a method for treating material subject to microbial contamination by administration  
of an effective antimicrobial amount of reptilian hemoglobin, or of the  $\alpha$  or  $\beta$  chains of  
this molecule, free of heme, fragments therefrom and combinations thereof. The  
invention also relates to compositions comprising such proteins, polypeptides or  
20 fragments.

### BACKGROUND OF THE INVENTION

Antibacterial peptides from natural sources have a long history. In 1939 Dubos  
demonstrated that a soil bacillus, subsequently identified as *B. brevis*, produced  
25 substances that could prevent pneumococcal infections in mice. Subsequently,  
Hotchkiss and Dubos purified two substances composed of amino acids and one of  
these, gramicidin, became available as a therapeutic agent. Subsequent studies on  
antimicrobial peptides have identified many active agents (1). (Within this application  
several publications are referenced by Arabic numerals within parentheses. Full citations  
30 for these references, listed in sequence, may be found at the end of the specification. All

of these references and any additional references cited within this application are herein incorporated by reference in their entirety.)

Many bacteria produce antimicrobial peptides (bacteriocins) and proteins; those released from Gram-negative bacteria are the more potent and have the wider spectrum of activity (2). The defensins are small antimicrobial peptides found in neutrophils, non-human macrophages and Paneth cells (3). Amphibian skin is a rich source of antimicrobial peptides, one of these, magainin, isolated from *Xenopus laevis*, currently is undergoing clinical trial (4,5). Plants form a variety of gene-encoded antimicrobial peptides including the phytoalexins, the PR proteins and the AMPs (6,7). Insects have been shown to synthesize bacteriocidal peptides and proteins such as cecropin obtained from the moth *Cecropia* (8,9,10) and the sarcotoxins obtained from the larvae of the flesh fly *Sarcophaga perigrina* (11). The hemocytes of the horse-shoe crab *Limulus* are the source of the tachyplesins and squalamine, an aminosteroid with antimicrobial activity, has been isolated from the shark, *Squalus acanthias* (12).

Thus, many antimicrobial substances lie within the families of "natural" antibiotics such as the cecropins, magainins, defensins, serprocidins and others. These substances are widely distributed in nature and provide an innate defense mechanism against infection in species ranging from insects to amphibians to mammals. Generally these substances are stored in cells, to be induced and secreted within the animal when challenged. Many act by disrupting the bacterial cell membrane selectively; many would be toxic to host cells as well, were they not sequestered (13). A number of these compounds have been proposed as being useful as antimicrobial agents (14,15).

Hemoglobin (MW=64,500) consists of four polypeptide chains and four heme prosthetic groups in which the iron atoms are in the ferrous state. The protein, called globin, consists of two  $\alpha$  chains and two  $\beta$  chains. In the alligator *Alligator mississippiensis*, the  $\alpha$  chain contains 141 amino acid residues and the  $\beta$  chain contains 146 residues. The amino acid sequence of the  $\alpha$  and  $\beta$  chains of alligator hemoglobin, is as follows:

3

$\alpha$  chain  
 1 15 16 30 31  
 45  
 VLSMEDKSNVKAIWG KASGHLEEYGAEALE RMFCAYPQTKIY  
 5 FPH  
 46 60 61 75 76  
 90  
 FDMSHNSAQIRAHGK KVFSALHEAVNHIDD LPGALCRLSELH  
 AHS  
 10 91 105 106 120 121  
 135  
 LRVDPVNFKFLAHCV LVVFAIHHPALSPE IHASLDKFLCAVS  
 AV  
 136 141  
 15 LTSKYR  
 $\beta$  chain  
 1 15 16 30 31 45  
 20 ASFDAHERKFIVDLW AKVDVAQCGADALSR  
 MLIVYPWKRRYFEHF  
 46 60 61 75 76 90  
 25 GKMCNAHDILHNSKV QEHGKKVLASFGEAV  
 KHLDNKGFANLSK  
 91 105 106 120 121 135  
 LHCEKFHVDPENFKL LGDIIIIVLAAHHPE DFSVECHAAFQKLVR  
 30  
 136 146  
 QVAAALAAEYH (SEQ ID NO: 1)

The structure of heme (ferroprotoporphyrin IX) is well known.

35 One heme group is bound to each polypeptide chain through a coordination bond  
 between the iron atom and the R group of a histidine residue. The sixth coordination bond

of the iron atom is available to bind oxygen. In addition, hemoglobin also transports  $H^+$ ,  $CO_2$ , and  $NO$ . The structure of heme is identical in all animals that have hemoglobin but the sequence of the globin chains varies considerably. In spite of this variation, the configuration of the tetramer is quite similar among species.

5       The interactions of hemoglobin with oxygen and carbon dioxide depend on the state of the heme and the residues surrounding it, as well as on regulation by heterotrophic ligands including  $H^+$ ,  $Cl^-$ ,  $CO_2$ ,  $HCO_3^-$  and 2,3-diphosphoglycerate. These ligands regulate the equilibrium between the high affinity state (the relaxed or R structure), and the low affinity tense state (or T structure). The stereochemistry of hemoglobin has been  
10 reviewed extensively (16,17).

      The hemoglobin of reptiles, Chelonia (turtles), Crocodilia (crocodilians) and Squamata (snakes and lizards) shows certain unique structural characteristics (18). In several species the hemoglobin tetramers have been found to form disulfide bridges with each other (19), although this may be largely an *in vitro* artifact. Also, high levels of  
15 methemoglobin (iron in the ferric state) have been found in spite of adequate levels of methemoglobin reductase (20). In most reptiles ATP is the primary regulator of oxygen affinity (21,22). In contrast, hemoglobin in crocodiles and alligators is unique (23), in that it is not responsive to organic phosphates but rather is regulated primarily by the bicarbonate ion which induces a decrease in oxygen affinity. The loss of sensitivity to  
20 phosphates apparently is caused by replacement of Pro or Ser for His at  $\beta$  NA2 and replacement by Ala for His at  $\beta$  H21. Also, the N-terminus of the chain of the hemoglobin from *Alligator mississippiensis* is blocked by an acetyl group (24,25,26). This alteration at the N-terminus permits hydrogen bonding with bicarbonate ions on the chains (25,26).

      Mammalian hemoglobin-based compositions have been developed for administration  
25 as blood substitutes. (27,28) These include chemically modified hemoglobin which

contains the oxygen-carrying heme group required for proper oxygen transport. While such modified hemoglobin-based compounds have been administered as blood substitutes, administration of unmodified hemoglobin, its heme free subunits or fragments or synthetic peptides therefrom has not previously been disclosed for this purpose or for other  
5 therapeutic uses. Indeed, the heme free  $\alpha$  and  $\beta$  subunits would not be utilized for the purpose of providing blood substitutes, as they are incapable of binding oxygen.

#### SUMMARY OF THE INVENTION

The present invention provides a method for killing bacteria or fungi comprising  
10 contacting the bacteria or fungi with an antimicrobially effective amount of reptilian hemoglobin protein, hemoglobin protein fragment or polypeptide fragments thereof selected from the group consisting of intact hemoglobin, heme-free hemoglobin  $\alpha$  chain, heme-free hemoglobin  $\beta$  chain, fragments of said proteins or polypeptide fragments thereof and combinations thereof.

15 The invention also provides a method for treating a subject having a bacterial or fungal infection comprising administering an antimicrobially effective amount of said protein, polypeptides and/or fragment compositions, to a method for treating material subject to bacterial or fungal contamination comprising applying to or admixing with said material an antimicrobially effective amount of said compositions, and to the use of said  
20 compositions for antimicrobial treatment of bacteria or fungi.

The invention additionally provides a pharmaceutical dosage form comprising an antimicrobially effective amount of said protein, polypeptide and/or fragment compositions and pharmaceutically acceptable carriers.

**BRIEF DESCRIPTION OF THE DRAWINGS**

- Figure 1: Activity of hemoglobin against *Escherichia coli* at pH 5.5 and 7.4.
- Figure 2: Activity of hemoglobin against *Pseudomonas aeruginosa* at pH 5.5.
- Figure 3: Activity of alpha chain against *Candida albicans* at pH 5.5.
- 5 Figure 4: Activity of alpha chain against *Escherichia coli* at pH 7.4.
- Figure 5: Activity of alpha chain against *Pseudomonas aeruginosa* at pH 5.5.
- Figure 6: Activity of beta chain against *Candida albicans* at pH 5.5.
- Figure 7: Activity of beta chain against *Escherichia coli* at pH 5.5.
- Figure 8: Activity of beta chain against *Pseudomonas aeruginosa* at pH 5.5.
- 10 Figure 9: Plot of C4-reverse phase HPLC analysis of alligator hemoglobin chains showing an early peak representing heme and subsequent broader peaks representing the alpha and beta chains.
- Figure 10: Tris-tricine-SDS-PAGE analysis of alligator hemoglobin showing coomassie blue staining of both the alpha and beta chains at 14.5 kDa.

15

**DETAILED DESCRIPTION OF THE INVENTION**

The subject of the invention is the antimicrobial activity of reptilian hemoglobin, its heme-free  $\alpha$  and  $\beta$  chains, fragments thereof and combinations thereof, and compositions comprising such peptides and fragments. Therapeutic applications for these substances

20 include use as broad spectrum topical and systemic antibacterial and antifungal agents, and agents exhibiting synergism with standard antibiotics.

This invention further provides compositions of matter and methods for treating microbial infections. More particularly, the compositions of this invention comprise hemoglobin or its  $\alpha$  or  $\beta$  chains, the latter without heme, derived from reptilian red blood

25 cells.



Further, these compositions also include fragments therefrom or combinations thereof. Bacteria against which the compositions have bactericidal activity include Gram-negative bacteria. Examples of such Gram-negative bacteria are *Escherichia coli* and *Pseudomonas aeruginosa*. Additionally, the compositions act as antimicrobial agents  
5 against fungi including yeast. In one embodiment of the invention, the yeast is *Candida albicans*.

Alligator hemoglobin exerts antimicrobial activity at pH 5.5 against fungi, such as *Candida albicans* and Gram-negative bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*. (Figures 1 through 3.)

10 Similar antimicrobial activity is exhibited at pH 5.5 for the heme-free  $\alpha$  and  $\beta$  chains of alligator hemoglobin. (Figures 4 to 6 for heme-free  $\alpha$  chain; and Figures 7 to 8 for the heme-free  $\beta$  chain.)

Hemoglobin from the garter snake is also active.

Although any mechanism proposed to account for the action of these peptides  
15 should not be considered limiting, it may be that the antimicrobial activity is contributed from the unique structure of the peptides which may form pores inside the membrane of the microorganisms. Because of similarities in the structure and configuration of hemoglobins from a variety of reptiles, it is likely that hemoglobin, its  $\alpha$  and  $\beta$  chains and fragments thereof obtained from sources other than alligator and snake will exert significant  
20 antimicrobial activity. The invention thus also encompasses hemoglobin tetramers and their constituent heme-free monomers from other reptiles.

The compositions of the invention may be used therapeutically, as preservatives or as disinfectants. This invention thus comprises a method for antimicrobially treating bacteria or fungi. This method comprises exposing the bacteria or fungi to an  
25 antimicrobially effective amount of one of the compositions described herein according to

any and each of the technologies described herein. When carrying out the method, the compositions are typically dissolved in an appropriate buffer. Examples of appropriate buffers are known in the art and include phosphate buffer (for fungi) or phosphate buffered saline at suitable values of pH.

5       The invention further provides a pharmaceutical composition useful for treating bacterial or fungal infections in a human or other mammalian subject by topical or systemic application. This pharmaceutical composition comprises an antimicrobially effective amount of one of the compositions of the invention and a pharmaceutically acceptable carrier. Suitable pharmaceutically acceptable carriers for topical, oral or systemic use are  
10 known in the art and are disclosed in the Pharmacopeia of the United States, The National Formulary and Pharmaceutical Science (8<sup>th</sup> Edition, Chapters 83, 84 and 89).

Depending on the specific application contemplated, the pharmaceutical composition provided by the present invention may be formulated as a solution, suspension, parenteral preparation, ointment, cream, lotion, spray, powder, tablet or capsule which is  
15 dosed, applied or admixed as appropriate. Parenteral preparations may include a vehicle such as specially distilled pyrogen-free water, phosphate buffer, or normal saline. Ointments, creams, lotions and sprays may include a carrier such as vegetable or mineral oil, white petrolatum, or a high molecular weight alcohol, i.e., possessing greater than 12 carbon atoms. Tablets or capsules may include diluents, e.g., lactose, binders, lubricants,  
20 e.g., stearic acid, and a disintegration aid, e.g., corn starch.

Each of the compositions of this invention may be combined with other antibiotics or antimicrobial agents, antiprotozoal agents, wound-healing agents and the like to enhance their activity or therapeutic spectrum.

Also provided is a method for treating a human or other mammalian subject having  
25 a bacterial or fungal infection which comprises administering to the subject an

antimicrobially effective amount of one of the pharmaceutical compositions of the present invention. The compositions can be administered to the subject by, for example, intravenous injection, intraperitoneal injection, orally, or in the form of an aerosol spray composition. Lipid vesicles or lipid emulsion preparations containing the peptides of the invention can also be used for administering the compositions. Specific modes of administration will depend on the pathogen to be targeted. The selection of the specific route of administration and the dose regimen is to be adjusted or titrated by the clinician according to methods known to said clinician in order to obtain the optimal clinical response. The amount to be administered is that amount which is antimicrobially effective.

10 The dosage administered will also depend on the characteristics of the subject being treated, e.g., the particular mammal treated, age, weight, health, types of concurrent treatment, if any, frequency of treatments, and therapeutic ratio. In the case of the treatment of human subjects, the antimicrobially effective amount will typically be in the range of from about 0.5 to 50 mg/kg body weight, and in the range of from about 0.5 - 5.0 mg/ml per dose.

15 Also provided is a method for using such peptides to prevent microbial contamination of food, i.e. as a preservative or to eliminate potential pathogens. For example, shell fish, meats and poultry products routinely harbor the growth of enteric pathogens. Such pathogens can be eliminated by treatment with an antimicrobially effective amount of the peptide compositions of the invention. Food crops, such as fruits

20 and vegetables could also be treated to eliminate post harvest spoilage. The peptides could be administered topically or through transgenic expression of a recombinant peptide of the invention. In the instance where the material to be preserved is mixed with the composition of the invention, an antimicrobially effective amount of the selected peptide is added by a simple blending method. The antimicrobially effective amount will typically be in the range

25 of from about 1500  $\mu$ g to 50 mg/kg of treated material. In the instance where the

10

compositions are administered topically, the antimicrobially effective amount will typically be in the range of from about 0.1 - 1.0 mg/cm<sup>2</sup>.

Additionally, the peptides of the invention can be used as disinfectant agents to sterilize or maintain microbe-free products. Such products can include baby wipes, diapers, 5 bandages, towelettes, make-up products, eyewash and contact lens solutions. The compositions of the invention may be administered to such products topically, in appropriate buffer or in liposome compositions. The antimicrobially effective amount to be administered will typically be in the range of from about 1500 µg to 50 mg/kg of treated material.

10 The following examples are intended to illustrate but not limit the present invention.

## EXAMPLES

### METHODS AND PREPARATIONS

#### Isolation of Reptilian Hemoglobin

15 Hemoglobin was isolated according to the following protocol. Venous blood of *Alligator mississippiensis* is withdrawn from the sagittal sinus into a syringe containing a concentration of heparin sufficient to prevent clotting. Blood (stored on ice) is equilibrated to room temperature and distributed in 2 polypropylene tubes (20 ml each) and gently mixed with an equal volume of 6% dextran. The suspension is left to stand until the red 20 cells have settled, at room temperature. This takes approximately 2 hrs. To promote better aggregation, approximately 1% dextran is added to each tube (4% dextran final concentration). Centrifugation at 1000g is used to separate red blood cell (pellet) from white blood cells/serum (supernatant). The red blood cell pellet is then washed 1x in PBS and stored at -20°C.

25 Hemoglobin from the garter snake *Thamnophis sirtalis* was purchased from Sigma

Chemicals.

**Purification of Hemoglobin (29)**

The frozen red blood cell pellet was thawed (7.5 mL) and mixed with 2.5 volumes  
5 of chilled deionized water. The resulting solution was hand shaken and kept on ice for 15  
min. The lysate was centrifuged at 20,000 x g for 1 hr. The upper 2/3 of this solution (20  
mL) was removed and passed through a mixed-bed ion-exchange column (15 mL, Bio-Rex  
RG501-X8, Bio-Rad). The effluent was then passed through a 0.22 µm filter (Millipore)  
by gravity. The filtrate was diluted with an equal volume of 0.1 M Tris-HCL (ph 7.8) to  
10 provide crude hemoglobin (Hb).

**Separation of α and β Chains from Hemoglobin (30).**

Crude Hb (50 µL) was injected onto a C4 reverse-phase HPLC column (YMC  
Protein-RP, 150 x 4.6 mm I.D.) and eluted with water/acetonitrile (mobile phase A; 80%  
15 H<sub>2</sub>O/20% AcCN/0.1 TFA; mobile phase B: 40% H<sub>2</sub>O/60% AcCN/0.1 TFA). The column  
was initially washed with 40% mobile phase B for 10 min, after which a linear gradient was  
run from 45% to 60% of B over 90 min. A second linear gradient from 55% to 95% of B  
was run over 10 min, after which the column was maintained at 95% B for 15 min. The  
flow rate of the column was 1 mL/min and eluted material was detected at 210 nm. The  
20 eluted volume was collected in 4 mL fractions which were concentrated under vacuum.  
The heme eluted as a sharp peak at 7.15 min, while the α and β subunits eluted as broad  
peaks centered at 54 min and 42 min, respectively (Figure 9).

**Tris/tricine-SDS-PAGE Analysis of Hemoglobin and its α and β Chains (31).**

25 Aliquots (20 µL) of Hb and its α and β chains were combined with 6X sample buffer

(4  $\mu$ L), heated at 95°C for 5 min and analyzed by SDS-PAGE (16.5% gel) using a tris/tricine buffer system. The bands were visualized by coomassie blue staining (0.1% coomassie blue G-250 in 50% methanol/10% acetic acid) for 1 h, followed by destaining of each gel (5% methanol/7% acetic acid) overnight (Figure 10).

5

#### Measurement of Antimicrobial Activity

Microbes utilized in the described assays were as follows:

*Pseudomonas aeruginosa* was strain PA01, *Escherichia coli* was strain MC4100 and *Candida albicans* was a clinical isolate from the Presbyterian Hospital.

10

#### Antibacterial Activity

##### A. Plate assay

The antibacterial activity of purified fractions, hemoglobin or its  $\alpha$  and  $\beta$  chains, was tested against bacteria, typified by *Escherichia coli*, maintained on Trypticase Soy Broth (TSB) agar plates. *Escherichia coli* is used as a representative of Gram-negative organisms. A single colony is inoculated into trypticase soy broth and grown to mid-exponential phase ( $OD_{600}=0.75$ ). The cultures are washed and diluted in 10 mM sodium phosphate buffer (NaPB) (pH 5.5 or 7.4), 150 mM NaCl (PBS) to a final concentration of  $2 \times 10^4$  colony forming units (CFU)/ml. Bacteria are incubated for 1 hour at 37°C with suitable concentrations of hemoglobin or the  $\alpha$  or  $\beta$  chains in PBS assay buffer. At the end of the assay, aliquots are diluted 1:10 in PBS and plated on agar plates with 0.8% soft agar to determine bacterial survival after overnight incubation at 37°C. Bactericidal activity is determined by calculating the decrease in colony forming units for bacteria incubated with hemoglobin or its  $\alpha$  or  $\beta$  chains as compared to bacteria incubated with buffer alone.

25

**B. Radial diffusion assays for antibiotic activity against *Pseudomonas aeruginosa* PA01**

A single colony of *Pseudomonas aeruginosa* PA01 is inoculated into trypticase soy broth and grown to mid-exponential phase ( $OD_{600} = 0.7$ ). The cultures are washed in NaPB pH 5.5 and dissolved to a final concentration of  $5 \times 10^7$  CFU/ml. A 0.2 ml aliquot of this bacterial suspension ( $10^7$  CFU) is added to 10 ml of autoclaved and cooled (to 42°C) NaPB, buffer, 1 % w/v of low electroendosmosis type agarose (Sigma). After mixing the bacteria in, the agarose is poured into Lab-Tek square Petri dishes to form a uniform 1 mm thick layer. Wells with a 2.7 mm diameter are punched in and filled with 4.5 ul of control or sample, the plates are incubated for 3 hours at 37°C, and overlaid with 10 ml of sterile agar maintained at 42°C. The overlay agar is 6% (w/v) TSB and 1% w/v Bacto-agar. After incubation for 18-24 hours at 37°C, the diameter of the clear zone surrounding the wells containing an antibacterial agent is measured.

**C. Radial diffusion assay for antibiotic activity against *Escherichia coli* MC4100**

A single colony of *Escherichia coli* MC4100 is inoculated into TSB and grown to mid-exponential phase ( $OD_{600} = 0.7$ ). The cultures are washed in NaPB, pH 5.5 and dissolved to a final concentration of  $5 \times 10^7$  CFU/ml. A 0.2ml aliquot of this bacterial suspension ( $10^7$  CFU/ml) is added to 10ml of autoclaved and cooled (to 42°C) NaPB buffer containing 0.02% Bovine Serum Albumin (BSA) and 0.02% Triton X100, 1% w/v of low electroendosmosis type agarose (Sigma). After mixing the bacteria in, the agarose is poured into Lab-Tek square Petri dishes to form a uniform 1 mm thick layer. Wells with a 2.7mm diameter are punched in and filled with 4.5 ul of control or sample, the plates are incubated for 3 hours at 37°C, and overlaid with 10 ml of sterile agar maintained at 42°C. The overlay agar is 6% (w/v) TSB and 1% w/v Bacto-agar. After incubation for 18-24

hours at 37°C, the diameter of the clear zone surrounding the wells containing an antibacterial agent is measured.

#### Antifungal Activity

##### 5 A. Plate assay

The antifungal activity of hemoglobin or its  $\alpha$  or  $\beta$  chains is tested against a fungus, as typified by *Candida albicans*, maintained on Sabouraud dextrose agar plates. The fungus *Candida albicans* used in these assays is a clinical isolate from Columbia Presbyterian Hospital, NY. A single colony is inoculated in Sabouraud dextrose broth and  
10 cultured for 16-18 hrs at 37°C. An aliquot of the overnight culture is inoculated in fresh broth and grown for 3 hrs to a density of  $7 \times 10^6$  / ml as determined with a counting chamber. The fungus culture is diluted to a final concentration of  $2 \times 10^4$  CFU/ml in NaPB, pH 5.5 and this suspension is incubated for 3 hrs with hemoglobin or its  $\alpha$  or  $\beta$  chains in NaPB, pH 5.5. Aliquots are diluted 1:10 in M63 minimal media and spread onto Sabouraud  
15 dextrose agar plates to determine surviving CFU after 20 hrs at 37°C.

##### B. Radial diffusion assay

The fungus is grown for 3 hrs from an overnight culture in Sabouraud dextrose broth, centrifuged at 10,000g for 10 min, washed twice in NaPB, pH 5.5, and resuspended  
20 in NaPB (pH 5.5) at a final concentration of  $4 \times 10^7$ /ml. A 0.1 ml aliquot of this fungal suspension ( $4 \times 10^6$  CFU) is added to 10 ml of autoclaved and cooled to (42°C) NaPB, pH 5.5 containing 1% w/v of low electroendosmosis type agarose (Sigma). After mixing the fungus in, the agar is poured into Lab-Tek square Petri dishes to form a uniform 1 mm thick layer. Wells with a 3 mm diameter are punched in and filled with 5  $\mu$ l of control or sample,  
25 the plates are incubated for 3 hrs at 37°C and overlaid with 10 ml of sterile agar maintained



at 42°C. The overlay agar is 2x Sabouraud agar. After incubation of 18-24 hrs at 37°C, the diameter of the clear zone surrounding the wells containing an antifungal agent is measured.

#### 5 Antimicrobial Activity of Peptides

Measurement of the antimicrobial activity of alligator hemoglobin and its heme-free  $\alpha$  and  $\beta$  chains was determined by radial diffusion assay. For the data provided in Figures 1-8, the ordinate shows diameter of clear zone expressed in arbitrary units where ten units = 1.0 mm (32). Abcissa shows protein concentration in  $\mu\text{g/ml}$ . MIC is estimated by linear  
10 extrapolation of data points to the x axis.

Measurement of the antimicrobial activity of alligator and garter snake hemoglobin against yeast also was determined by plate assay.

Each radial diffusion assay was performed with 3 experimental runs except where indicated. The radial diffusion assay is reliable and gives consistent results when used with  
15 the purified or semipurified compositions of the invention. The assay method was patterned after that of Lee et al (32).

#### Example 1

Antimicrobial activity for *Alligator mississippiensis* hemoglobin is summarized in  
20 Table 1, Example 1 and in Figures 1 through 3. The minimum effective concentration (MIC), expressed as  $\mu\text{g/ml}$ , at pH 5.5 was as follows: for *Escherichia coli*, 40-50  $\mu\text{g/ml}$  and *Pseudomonas aeruginosa*, 300  $\mu\text{g/ml}$ .

#### Examples 2 and 3

25 In addition, antimicrobial activity was exhibited by the  $\alpha$  and  $\beta$  chains of the

*Alligator mississippiensis* hemoglobin lacking the heme group. This activity is summarized in Table 1, Example 2 and Figures 4-6 for the  $\alpha$  chain and Table 1, Example 3 and Figures 7-8 for the  $\beta$  chain.

**TABLE 1**  
**ANTIMICROBIAL ACTIVITY**

**Radial Diffusion Assays**  
**Alligator Hemoglobin and heme-free  $\alpha$  and  $\beta$  Subunits**  
**Minimum Inhibitory Concentration (MIC) in  $\mu\text{g/ml}$**

Microorganism	Hemoglobin Example 1	$\alpha$ chains Example 2	$\beta$ chains Example 3
<i>Candida albicans</i>	-	20-30	90-150
<i>Escherichia coli</i>	40-50	10-20	20-100
<i>Pseudomonas aeruginosa</i>	300	25-100	350

Lack of effect indicated by (-)

\*\*\*\*\*

#### Example 4

Hemoglobin for the alligator, *Alligator mississippiensis*, was active against *Candida albicans* at an LD<sub>50</sub> concentration ranging from 4-8  $\mu\text{g/ml}$  in a plate assay.

#### Example 5

Hemoglobin from the garter snake, *Thamnophis sirtalis*, was active against *Candida albicans* at an LD<sub>50</sub> concentration of 2.5  $\mu\text{g/ml}$  in a plate assay.

\*\*\*\*\*

The foregoing examples demonstrate experiments performed and contemplated

by the present inventors in making and carrying out the invention. It is believed that these examples include a disclosure of techniques which serve to demonstrate the practice of and usefulness of the invention. It will be appreciated by those skilled in the art that various changes may be made in the embodiments and techniques exemplified

5 without departing from the scope of the invention.

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**WHAT IS CLAIMED IS:**

1                   1.     A method for killing bacteria or fungi comprising contacting the  
2 bacteria or fungi with an antimicrobially effective amount of reptilian hemoglobin  
3 protein, hemoglobin protein fragment or polypeptide fragments thereof selected from  
4 the group consisting of intact hemoglobin, heme-free hemoglobin  $\alpha$  chain, heme-free  
5 hemoglobin  $\beta$  chain, fragments of said proteins or polypeptide fragments thereof and  
6 combinations thereof.

1                   2.     The method according to claim 1, wherein said hemoglobin  
2 protein, hemoglobin protein fragment or polypeptide fragments are derived from  
3 alligator.

1                   3.     The method according to claim 2, wherein the alligator is  
2 *Alligator mississippiensis*.

1                   4.     The method according to claim 1, wherein the bacteria are Gram-  
2 negative bacteria.

1                   5.     The method according to claim 4, wherein the Gram-negative  
2 bacteria are selected from the group consisting of *Escherichia coli* and *Pseudomonas*  
3 *aeruginosa*.

1                   6.     The method according to claim 1, wherein the fungi are *Candida*  
2 *albicans*.



1                   7.     The method according to claim 1, wherein the reptile is  
2     *Thamnophis sirtalis*.

1                   8.     A method for treating a subject having a bacterial or fungal  
2     infection which comprises administering to the subject an antimicrobially effective  
3     amount of reptilian hemoglobin protein, hemoglobin protein fragment or polypeptide  
4     fragments thereof selected from the group consisting of intact hemoglobin, heme-free  
5     hemoglobin  $\alpha$  chain, heme-free hemoglobin  $\beta$  chain, fragments of said proteins or  
6     polypeptide fragments thereof and combinations thereof

1                   9.     The method according to claim 8, wherein said hemoglobin  
2     protein, hemoglobin protein fragment or polypeptide fragments are derived from  
3     alligator.

1                   10.    The method according to claim 9, wherein the alligator is  
2     *Alligator mississippiensis*.

1                   11.    The method according to claim 8, wherein the bacteria are Gram-  
2     negative bacteria.

1                   12.    The method according to claim 11, wherein the Gram-negative  
2     bacteria are selected from the group consisting of *Escherichia coli* and *Pseudomonas*  
3     *aeruginosa*.

1                   13.    The method according to claim 8, wherein the fungi are *Candida*

2 *albicans*.

1                   14.    The method according to claim 8, wherein the reptile is  
2 *Thamnophis sirtalis*.

1                   15.    A method for treating material subject to bacterial or fungal  
2 contamination, comprising applying or admixing with said material an antimicrobially  
3 effective amount of reptilian hemoglobin protein, hemoglobin protein fragment or  
4 polypeptide fragments thereof selected from the group consisting of intact hemoglobin,  
5 heme-free hemoglobin  $\alpha$  chain, heme-free hemoglobin  $\beta$  chain, fragments of said  
6 proteins or polypeptide fragments thereof and combinations thereof.

1                   16.    The method according to claim 15, wherein said hemoglobin  
2 protein, hemoglobin protein fragment or polypeptide fragments are derived from  
3 alligator.

1                   17.    The method according to claim 16, wherein the alligator is  
2 *Alligator mississippiensis*.

1                   18.    The method according to claim 15, wherein the bacteria are  
2 Gram-negative bacteria

1                   19.    The method according to claim 18, wherein the Gram-negative  
2 bacteria are selected from the group consisting of *Escherichia coli* and *Pseudomonas*  
3 *aeruginosa*.

1                   20.    The method according to claim 15, wherein the fungi are  
2    *Candida albicans*.

1                   21.    The method according to claim 15, wherein the reptile is  
2    *Thamnophis sirtalis*.

1                   22.    A pharmaceutical dosage form comprising an antimicrobially  
2    effective amount of mammalian hemoglobin protein, hemoglobin protein fragment or  
3    polypeptide fragments thereof selected from the group consisting of intact hemoglobin,  
4    heme-free hemoglobin  $\alpha$  chain, heme-free hemoglobin  $\beta$  chain, fragments of said  
5    proteins or polypeptide fragments thereof and combinations thereof, and a  
6    pharmaceutically acceptable carrier.

1                   23.    The pharmaceutical dosage form according to claim 22, wherein  
2    said hemoglobin protein, hemoglobin protein fragment or polypeptide fragments are  
3    derived from alligator

1                   24.    The pharmaceutical dosage form according to claim 23, wherein  
2    said alligator is *Alligator mississippiensis*.

1                   25.    The pharmaceutical dosage form according to claim 22, wherein  
2    said hemoglobin protein, hemoglobin protein fragment or polypeptide fragments are  
3    derived from *Thamnophis sirtalis*.

- 1                   26.    The use of a reptilian hemoglobin protein, hemoglobin protein  
2    fragment or polypeptide fragments thereof selected from the group consisting of intact  
3    hemoglobin, heme-free hemoglobin  $\alpha$  chain, heme-free hemoglobin  $\beta$  chain, fragments of  
4    said proteins or polypeptide fragments thereof and combinations thereof, and a  
5    pharmaceutically acceptable carrier for antimicrobial treatment of bacteria or fungi.

FIG. 2

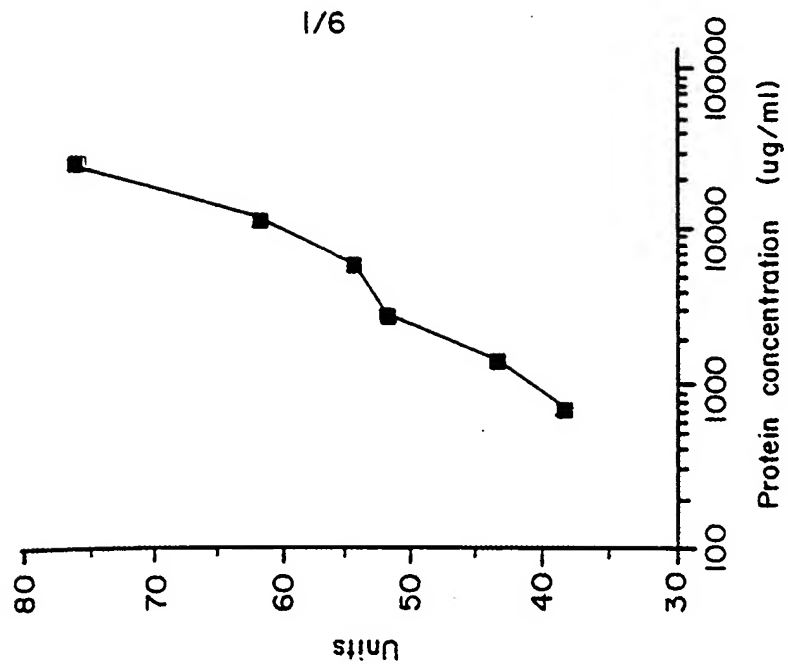
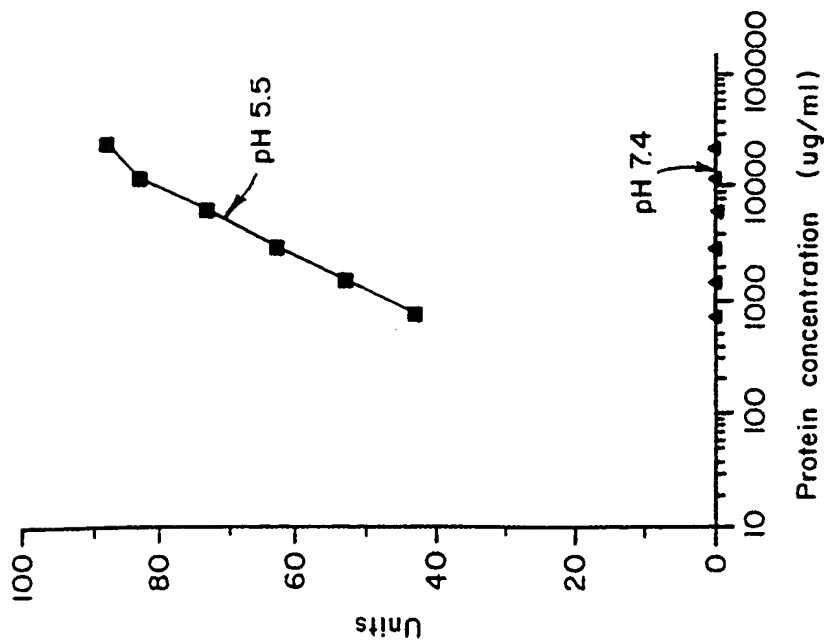


FIG. 1



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FIG. 4

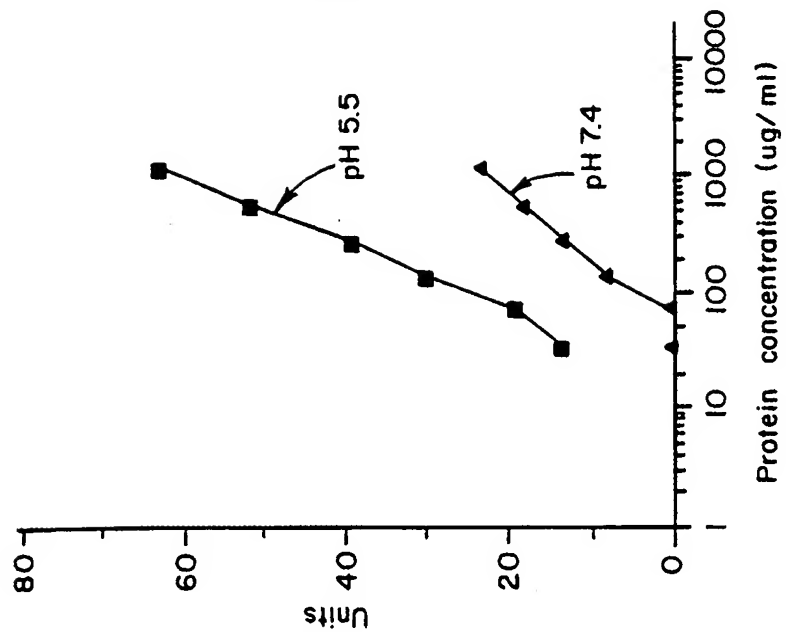
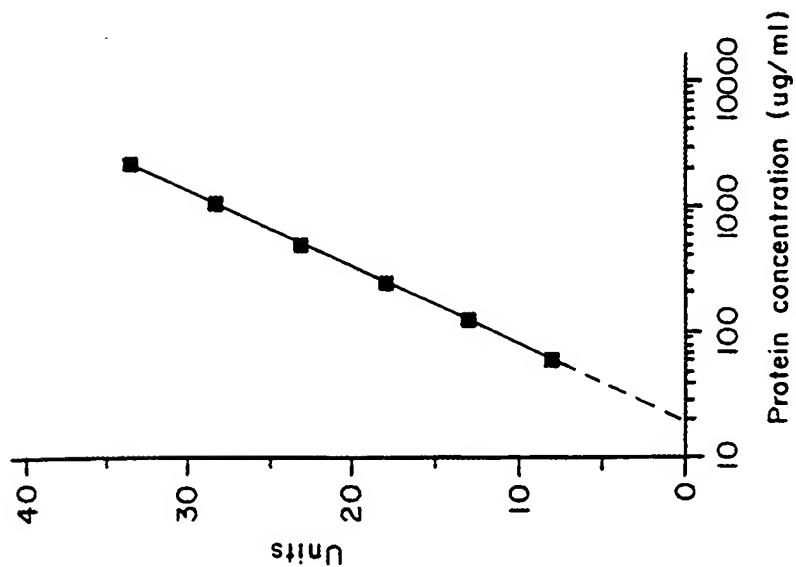


FIG. 3



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FIG. 6

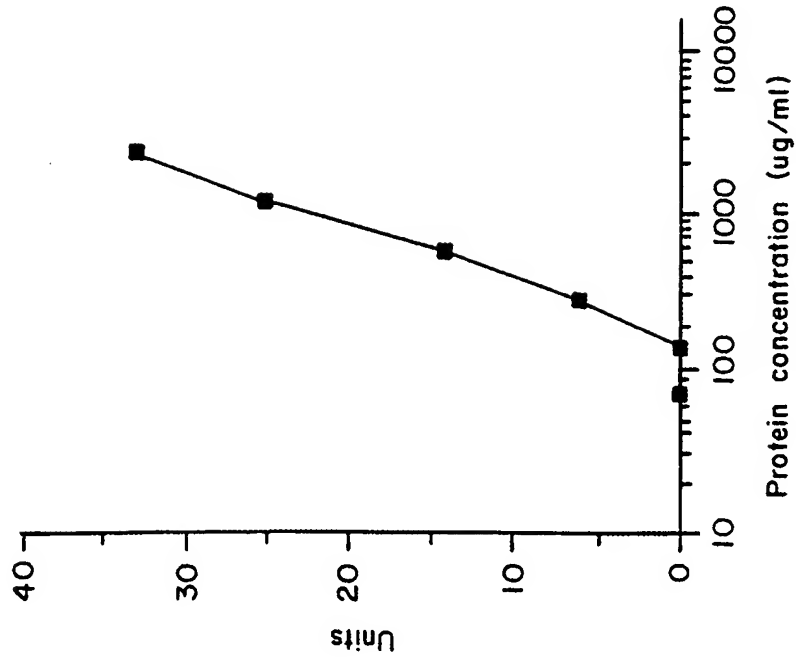
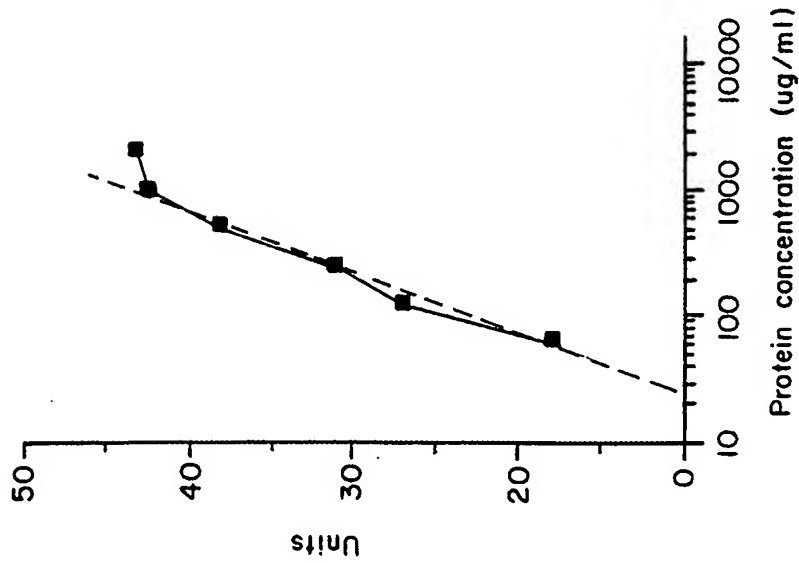


FIG. 5



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FIG. 8

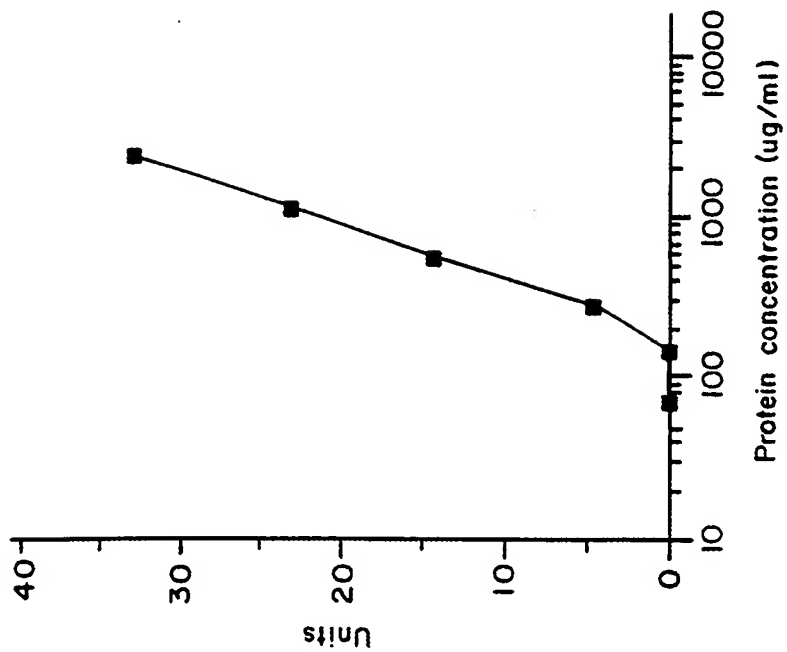
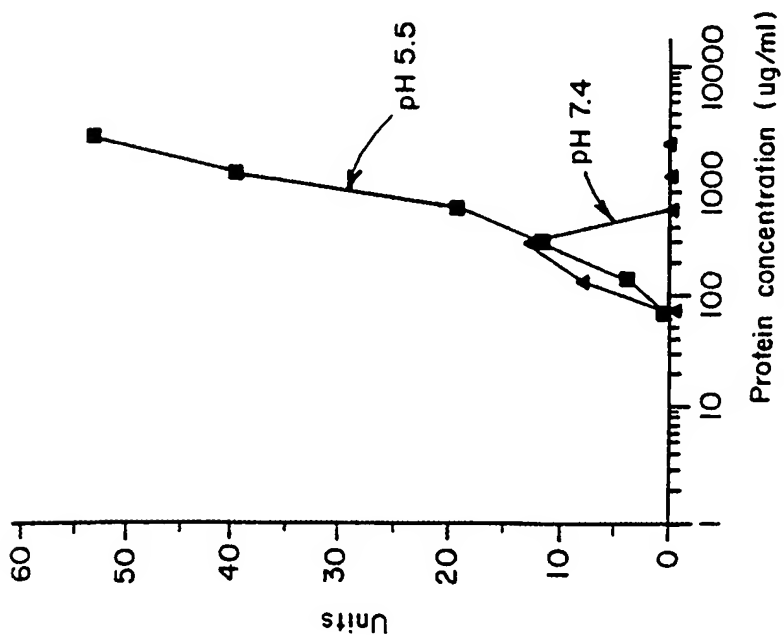


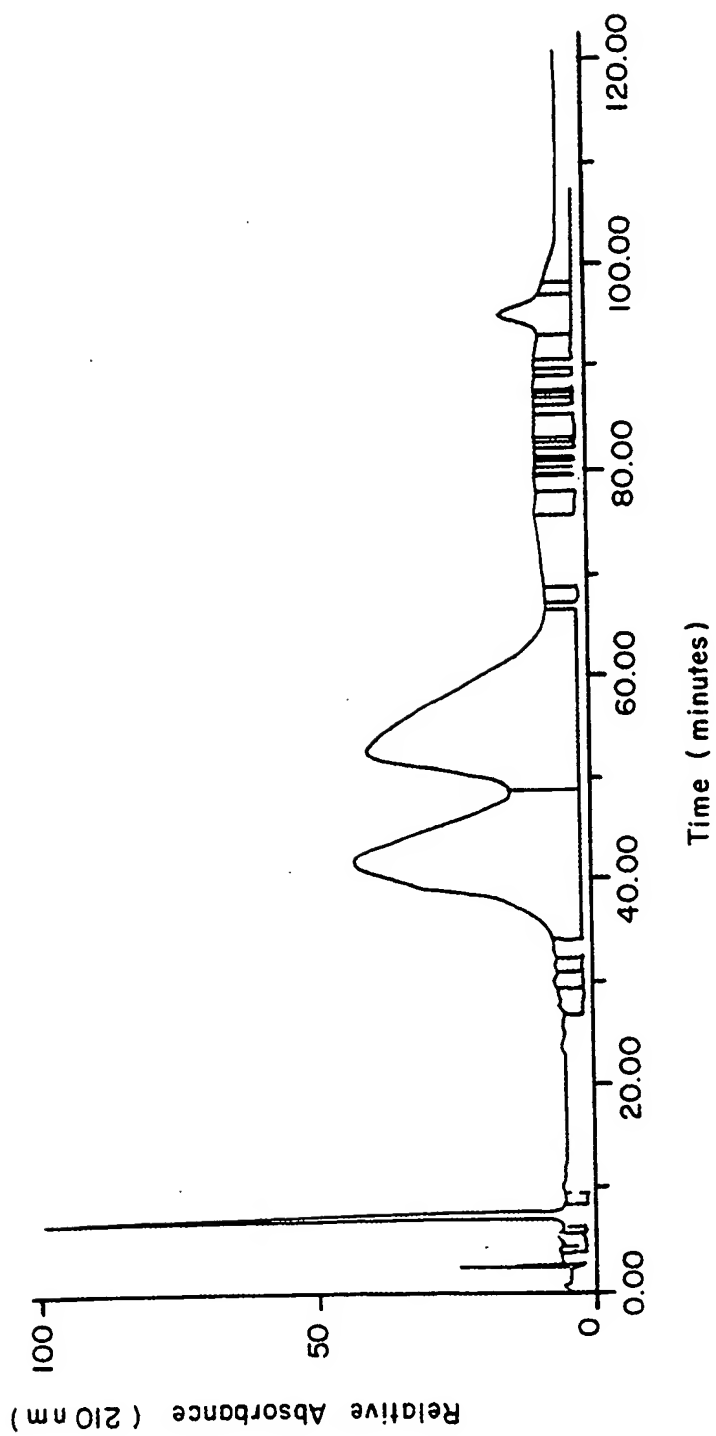
FIG. 7





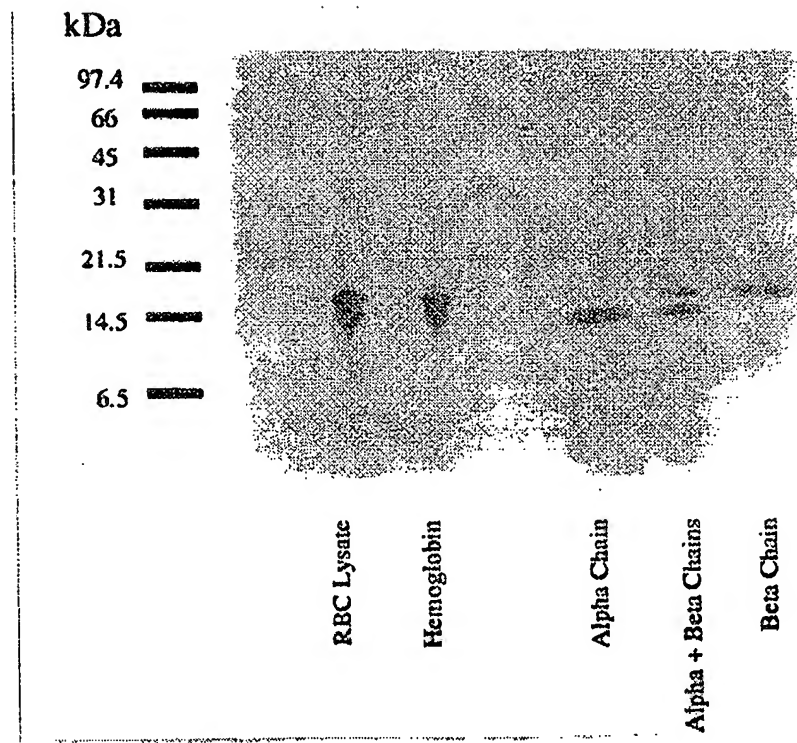
5/6

FIG. 9



SUBSTITUTE SHEET (RULE 26)

FIG. 10



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/16659

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) :A61K 31/74, 35/14, 38/00, 38/16 US CL :Please See Extra Sheet. According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 514/2, 6, 12, 21; 530/324, 380, 385, 386, 827, 829; 424/78.07, 529, 530 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,380,664 A (CARVER ET AL) 10 January 1995, col. 3, lines 39 to col. 4, lines 12; and claims 3-4.	15-17 and 26
A	NICOLAS ET AL. Peptides as Weapons Against Microorganisms in the Chemical Defense System of Vertebrates. Annual Review of Microbiology. 1995. Vol. 49. pages 277-304, especially pages 277-279.	1-26
A	PERUTZ ET AL. Allosteric Regulation of Crocodilian Haemoglobin. Nature. 25 June 1981, Vol. 291, pages 682-684.	1-26
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *A* document member of the same patent family		
Date of the actual completion of the international search 08 OCTOBER 1998		Date of mailing of the international search report 23 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer ABDEL A. MOHAMED Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/16659

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

514/2, 6, 12, 21; 530/324, 380, 385, 386, 827, 829; 424/78.07, 529, 530

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, BIOSIS, EMBASE, WPIDS

search terms: kill or destroy or disinfect, bacteri? or fungus or fungi or microb?, hemoglobin reptil? or crocodil? or alligator, protein or polypeptide, gram negative or escherichia or pseudomonas or candida, administer? or treat? or therapeutic? or pharmaceut?